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OIPE JC42 MAR 25 2004 PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No.:	**P-576 (TI-0022)**
Inventors:	**Huber et al.**
Serial No.:	**09/770,410**
Filing Date:	**January 25, 2001**
Examiner:	**Ernest G.Therkorn**
Group Art Unit:	**1723**
Title:	**Method and Apparatus for Separating Polynucleotides Using Monolithic Capillary Columns**

DECLARATION UNDER 37 C.F.R. §1.131

1. I, Christian Huber, together with Andreas Premstaller and Herbert Oberacher, am an inventor of the above referenced U.S. Patent Application Serial No. 09/770,410.

2. I am familiar with the teachings of the paper by Gusev et al. published in Issue 855 of the Journal of Chromatography on September 3, 1999, hereinafter referred to as Gusev.

3. Gusev describe a porous monolithic packing prepared with polystyrene-divinylbenzene support which is covalently attached to a fused silica capillary inner wall treated with a coupling agent trimethoxysilyl propyl methacrylate to provide anchoring sites for grafting of the polymer to the silica surface. The median pore radius for a monolithic sample prepared with ethanol is, as estimated by Gusev, about 5 micrometers.

4. Our invention referenced above, teaches a device for separating a mixture of polynucleotides by ion pair-reversed phase-high performance liquid chromatography. The device comprises a polymeric monolith having non-polar chromatographic surfaces. The monolith comprises an underivatized poly-(styrene/divinylbenzene) matrix and is contained in within a tube having an inner diameter in the range of 1 to 1000 micrometers.

5. Laboratory protocol notebooks regarding experiments related to this invention were kept by my then Ph.D student, Andreas Premstaller.

6. Andreas Premstaller worked for me in my laboratory and under my direct supervision during 1998 and 1999.

7. According to laboratory protocol notebooks, the first synthesis of PS/DVB monolith using decanol and tetrahydrofuran as porogens was performed on August 6, 1998. We then succeeded in a first separation of proteins (lysosyme from beta-lactoglobulin B) in a monolithic column on August 25, 1998. The first successful separation of oligonucleotides on a PS/DVB monolith synthesized with decanol/THF as porogens was February 9, 1999.

8. We were able to fully practice our invention described in the above referenced patent application prior to the date of the publication of the Gusev paper. A copy of the relevant laboratory notebook pages hereby accompanies my declaration.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Christian Huber

Christian Huber, Ph. D

6.8.98

Monolithsynthese mit THF M11

C10OH nicht zu 98

Nr.	Datum	Kapillare ID/OD [µm]	Polymerisationsmischung Styrol [ml]	DVB [ml]	AIBN [g]	C12OH [ml]	THF [ml]	Temperatur [°C]
M11_1	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	3.00	0.00	70, TS
M11_2	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.90	0.10	70, TS
M11_3	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.80	0.20	70, TS
M11_4	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.70	0.30	70, TS
M11_5	05.08.98	320/450 WBH06A, 20 cm, vs	1.00	1.00	0.050	2.60	0.40	70, TS

THF sollte besser Lm des Harzes sein und ähnlicher als Toluol

THF destilliert, da mit Radikalfänger (Phenol) stabilisiert.

Ausgangsmaterial: VS 3.8.98 trocken
THF dest.

Start: 6.8.98 10^{00}h   T = 70°C

Ende: 7.8.98 12^{00}h   T = 70°C

7.8.98

M11_1 16 cm

Fluß [µl/min]	Gegendruck [bar]	[bar/cm]
5	1	0.06
10	1	0.06
25	4	0.25
50	7	0.44
100	14	0.88
150	21	1.31
200	28	1.75
k [bar cm^{-1} µl^{-1}min]		0.008712

M11_2 15 cm

Fluß [µl/min]	Gegendruck [bar]	[bar/cm]
10	1	0.07
25	4	0.27
50	8	0.53
100	14	0.93
150	20	1.33
200	25	1.67
k [bar cm^{-1} µl^{-1}min]		0.008303

M11_3 15 cm

Fluß [µl/min]	Gegendruck [bar]	[bar/cm]
10	1	0.07
25	3	0.20
50	6	0.40
100	11	0.73
150	14	0.93
200	19	1.27
k [bar cm^{-1} µl^{-1}min]		0.006114

M11_4 16 cm

Fluß [µl/min]	Gegendruck [bar]	[bar/cm]
5	9	0.56
10	14	0.88
25	32	2.00
50	68	4.25
100	126	7.88
150	180	11.25
k [bar cm^{-1} µl^{-1}min]		0.074602

M11_5 16 cm 200bar Gegendruck

Fluß [µl/min]	Gegendruck [bar]	[bar/cm]
1	21	1.31
2	33	2.06
3	49	3.06
4	63	3.94
5	77	4.81
7	92	5.75
10	170	10.63
12	184	11.50
k [bar cm^{-1} µl^{-1}min]		0.963149

Anteil THF [% Porogen]	Steigung k [bar cm^{-1} µl^{-1}min]
0.0%	0.00871
3.3%	0.00830
6.7%	0.00611
10.0%	0.07460
13.3%	0.96315

Mikroskop: SS Stahlteil: 450µ   1 Teil → 8µm

M11_1 kleine Poren ~3µ, sehr porös.

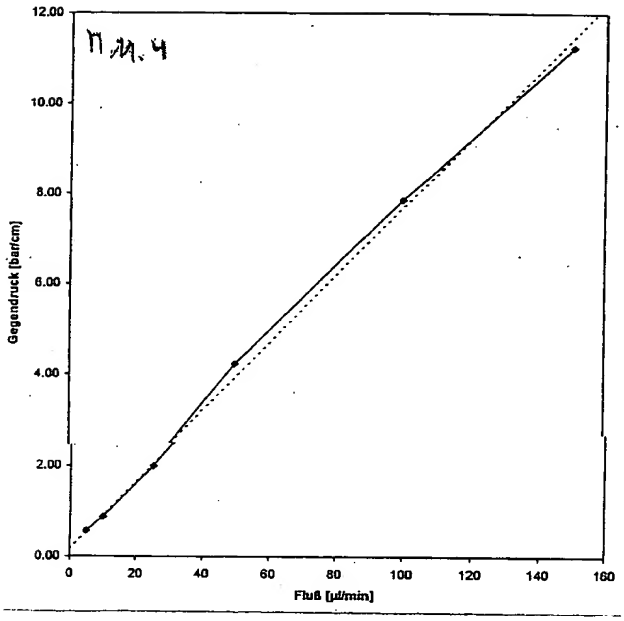
M11_2 große Poren, recht gleichmäßig

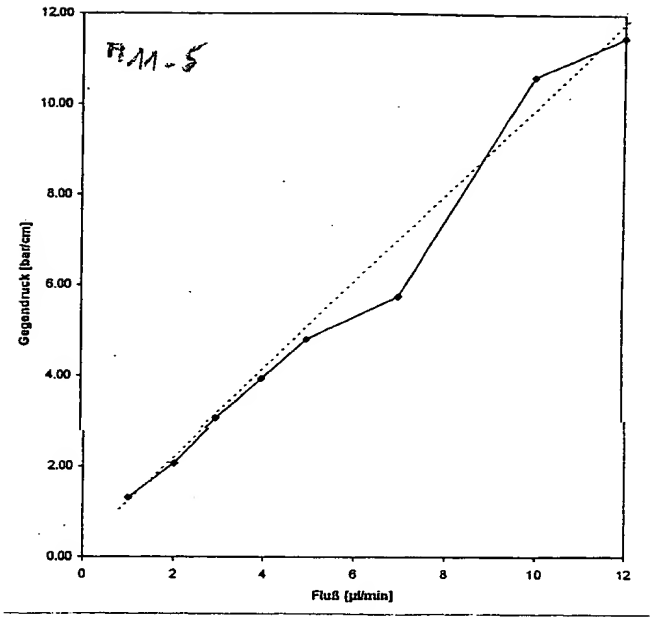
M11_3 große Poren, einzelne Kanäle (µm-Größe)

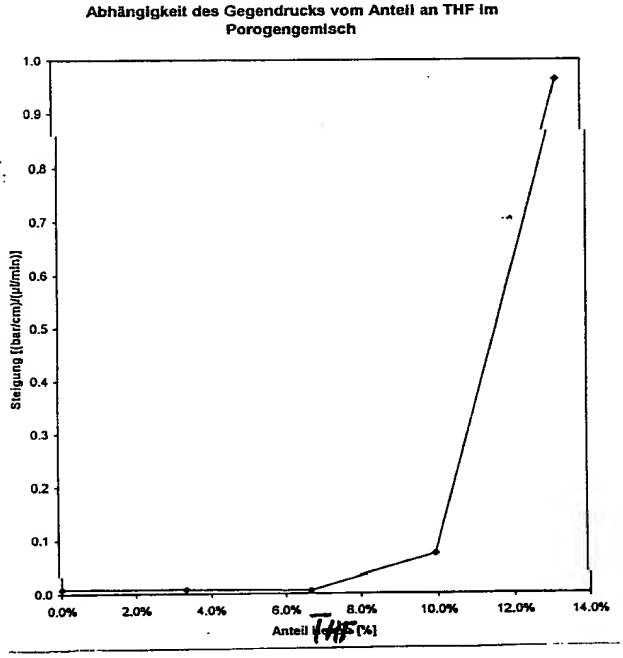
M11_4 sehr dicht, keine Risse, porös, aber nicht gut zu sehen

M11_5 keine Mikrostruktur zu erkennen.

ab







Als nächstes Bereich zwischen MM.4 und MM.5 genau untersuchen.

Für HPLC MM.4 und MM.5 testen.

25.08.98

M 11-5 nm 6.8.98

kein Spitz 240 bar / 5 µl/min

Fil. AP80875.SIP
GYNKOSOFT

SYKAM. 130 µl/min → Split → 4.6 µl/min
2 min 11 sec / 10 µl
2 min 30 sec 4 µl/min

Equilibrium:
(A) H_2O, 0.1% TFA
(B) ACN, 0.1% TFA
50% A / 14.50 –

Edelstahl - T-Stück ~~Teflon~~ regelbares T-Stück
10 µl / 2 min 15 sec $\frac{10}{2.25}$ 4.44 µl/min

Bestätigung:
Thiohormotop 0.05% in H_2O 50% ACN, 0.1% TFA
p ≙ 200 bar

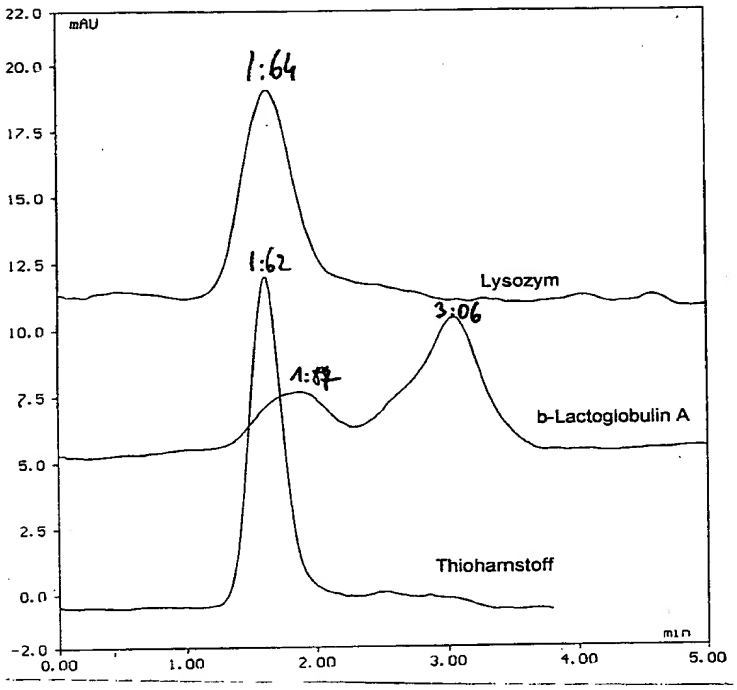
100% H_2O, 0.1% TFA: Protein zeigt kein Peak → klebt an Kapillare?
Thiohormotop near ca. 1.50 min

50% ACN, 0.1% TFA: Protein gleichzeitig mit Thiohormotop: keine Retention
RIBA

27.8.98

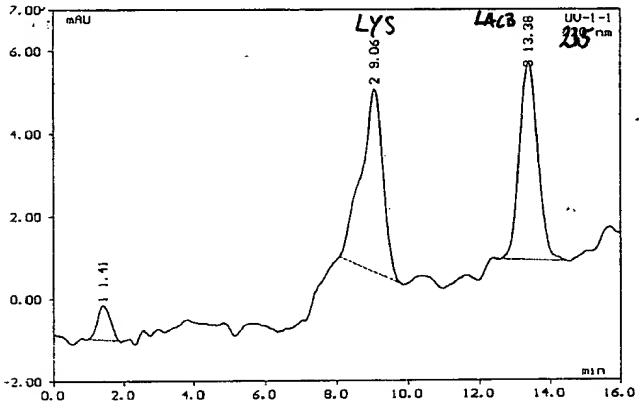
50% ACN, 0.1% TFA: Protein quantitativ eluiert als Peak.
LACA.
LYS keine Retention

Neu 40% ACN



50% ACN, 0.1% TFA
1 µl 2 mg/ml Protein
Retention von LacA bei 50% ACN

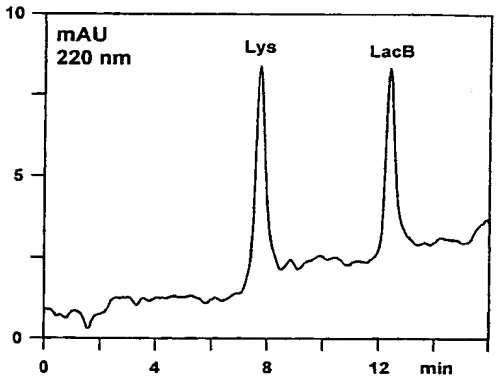
```
DP.Integration              SYS1 - C:A980825.SMP                    Page   2
PSDVB 100x0.32mm, C12OH/THF-Porogen, M11_5 98086                    1998-08-27/20:13
-------------------------------------------------------------------------------
LysLacB 1mg/ml                    UV-1-1                        1998-08-27
Modified. H2O/30-60,15minACN/0.1%TFA,4.5/130ul/min,25°C         GynkoSoft V5.50
-------------------------------------------------------------------------------
Smp. No/Pos: 35/1              Control:                 Standard:     --------
Sample Type: Integration       Signals: ANDI1.SIG       Inject:       20.0 uL
Acquisition: 1998-08-27/19:53  Report:                  Dil. Fact.:   1.00000
Method:      DEFAULT.INT       P-Table:                 Weight:       1.00000
-------------------------------------------------------------------------------
```



No.	Ret.Time min	Type	Area mAU*min	Height mAU	Half Width min	Base Width min	Plates
1	1.414	MB	3.551e-1	0.84	0.415	0.720	64
2	9.060	BMB	3.170e+0	4.46	0.627	0.972	1156
3	13.379	BMB	2.920e+0	4.82	0.567	0.988	3080
---	------	---	6.445e+0	10.12	--------	--------	--------

Effektive Proteintrennung:
Lys, LacB je 1 mg/ml, 20 nl inj.
30 - 60% ACN / 15 min, 0.1% TFA
4.5 / 130 µl/min
215 nm

A980825 -36



Separation of proteins in a monolithic capillary column

Column, PS-DVB (monolith, 100 x 0.32 mm); chromatographic conditions, mobile phase, (A) H_2O, 0.1% TFA, (B) ACN, 0.1% TFA, linear gradient, 30-60% B in 15 min; flow rate, 4.5 µl min^{-1}; temperature, 25 °C; detection, UV, 220 nm; sample, lysozyme, β-lactoglobuline B, 20 ng each.

Versuch einer Trennung von Oligonucleotiden im Monolithen M13_5

M13-5

l = 82 mm , id = 200µm

Eluenten: A: 50mM TEAA pH6.8

B: 50mM TEAA 20% ACN pH6.8

Temperatur: 50°C

Splitkapillare TSP025375, 6 cm   Flow 120 / 3.3 µl/min / 94 bar

File: A990209.STR

Trennung von dT_8, dT_{16}

Peakbreite mit Gradient 0-100% B/10min. 0.11 min

= 6.6 s

Trennung von dT_{12-18}

verschiedene Gradienten versucht.

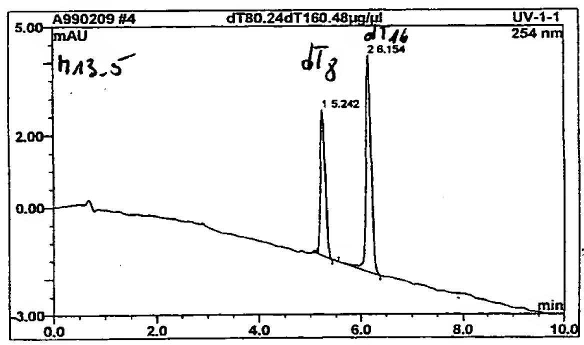
gute Trennung: 30-50% B/10min

6 - 10% ACN/10min

Operator:c72551 Timebase:A990209 Sequence:A990209 — Page 4-1, 10.2.1999 2:35 PM

4	dT80.24dT160.48µg/µl
0-100%B/10min;A:50mMTEAApH6.8,B:50mMTEAA20%ACNpH6.8;120/3.3µl/min;D:2min;50°C	

Sample Name:	dT80.24dT160.48µg/µl	*Injection Volume:*	20.0 nl
Control Program:		*Channel:*	UV-1-1
Quantif. Method:	OLIGO1	*Recording Time:*	09.02.99 19:00

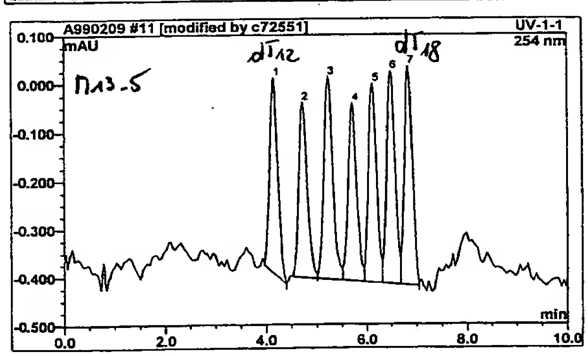


No.	Ret.Time min	Area mAU*min	Height mAU	Half Width min	Plates (EP)	Asymmetry (AIA)
1	5.242	0.450	4.053	0.108	13593	1.303
2	6.154	0.744	6.008	0.111	16926	1.331
Total:		1.194	10.061			

Operator:c72551 Timebase:A990209 Sequence:A990209 — Page 11-1, 10.2.1999 2:34 PM

11	dT12-18 0.25µg/µl
30-50%B/10min;A:50mMTEAApH6.8,B:50mMTEAA20%ACNpH6.8;120/3.3µl/min;D:2min;50°C	

Sample Name:	dT12-18 0.25µg/µl	*Injection Volume:*	20.0 nl
Control Program:		*Channel:*	UV-1-1
Quantif. Method:	OLIGO1	*Recording Time:*	09.02.99 21:29



No.	Ret.Time min	Area mAU*min	Height mAU	Half Width min	Plates (EP)	Asymmetry (AIA)
1	4.156	0.075	0.405	0.175	3142	1.050
2	4.707	0.071	0.384	0.178	3889	1.551
3	5.224	0.088	0.420	0.192	4101	1.246
4	5.709	0.073	0.370	0.180	5552	1.084
5	6.122	0.082	0.412	0.189	5813	n.a.
6	6.483	0.086	0.441	0.182	7042	n.a.
7	6.835	0.082	0.454	0.171	8886	n.a.
Total:		0.556	2.866			